Eur. J. Biochem. **271**, 1465–1475 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04056.x

S-Stereoselective piperazine-2-*tert*-butylcarboxamide hydrolase from *Pseudomonas azotoformans* IAM 1603 is a novel L-amino acid amidase

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An amidase acting on (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide was purified from *Pseudomonas azotoformans* IAM 1603 and characterized. The enzyme acted *S*-stereoselectively on (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide to yield (*S*)-piperazine-2-carboxylic acid. N-terminal and internal amino acid sequences of the enzyme were determined. The gene encoding the *S*-stereoselective piperazine-2-*tert*-butylcarboxamide amidase was cloned from the chromosomal DNA of the strain and sequenced. Analysis of 2.1 kb of genomic DNA revealed the presence of two ORFs, one of which (*laaA*) encodes the amidase. This enzyme, LaaA is composed of 310 amino acid residues (molecular mass 34 514 Da), and the deduced amino acid sequence exhibits significant similarity to hypothetical and functionally characterized proline iminopeptidases from several bacteria. The *laaA* gene modified in the nucleotide sequence upstream from its start codon was overexpressed in *Escherichia coli*. The activity of the recombinant LaaA enzyme in cell-free extracts of *E. coli* was 13.1 units·mg^{-1} with L-prolinamide as substrate. This enzyme was purified to electrophoretic homogeneity by ammonium sulfate fractionation and two column chromatography steps. On gel-filtration chromatography, the enzyme appeared to be a monomer with a molecular mass of 32 kDa. It had maximal activity at 45 °C and pH 9.0, and was completely inactivated in the presence of phenylhydrazine, Zn^{2+}, Ag^{+}, Cd^{2+} or Hg^{2+}. LaaA had hydrolyzing activity toward L-amino acid amides such as L-prolinamide, L-proline-*p*-nitroanilide, L-alaninamide and L-methioninamide, but did not act on the peptide substrates for the proline iminopeptidases despite their sequence similarity to LaaA. The enzyme also acted *S*-stereoselectively on (*R*,*S*)-piperidine-2-carboxamide, (*R*,*S*)-piperazine-2-carboxamide and (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide. Based on its specificity towards L-amino acid amides, the enzyme was named L-amino acid amidase. *E. coli* transformants overexpressing the *laaA* gene could be used for the *S*-stereoselective hydrolysis of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide.

Keywords: amidase; L-prolinamide; piperazine-2-*tert*-butylcarboxamide; *Pseudomonas azotoformans*.

Amidases (acylamide amidohydrolases, EC 3.5.1.4) catalyze the hydrolysis of the carboxyl amide bonds to liberate carboxylic acids and ammonia. Recently, various kinds of stereoselective amidases from microbial origin have been reported and received much attention because of their potential use for the industrial production of optically active compounds [1–3]. *S*-Enantiomer-selective amidases from *Brevibacterium* sp. R312 [4], *Pseudomonas chlororaphis* B23 [5] and *Rhodococcus rhodochrous* J1 [6] were found to be involved in nitrile metabolism with genetically linked nitrile hydratases. *S*- and *R*-enantiomer-selective amidases, which seemed not to be related to the nitrile metabolism, were also found in *Agrobacterium tumefaciens* d3 [7] and *Comamonas acidovorans* KPO-2771-4 [8], respectively. These enantiomer-selective amidases can be used for the production of optically active 2-arylpropionic acids, the nonsteroid anti-inflammatory drugs, from the corresponding racemic amides. *S*-Stereoselective amino acid amidases from *Pseudomonas putida* ATCC 12633 [9], *Ochrobactrum anthropi* NCIMB 40321 [10] and *Mycobacterium neoaurum* ATCC 25795 [11], and the *R*-stereoselective amino acid amidases from *O. anthropi* C1-38 [12,13], *O. anthropi* SV3 [14], *Arthrobacter* sp. NJ-26 [15] and *Brevibacillus borstelensis* BCS-1 [16] were found to be useful for the production of enantiomerically pure amino acids and their derivatives from the corresponding racemic amino acid amides. The genes coding for the above amidases have been isolated and their primary structures revealed, except for the *S*-stereoselective amino acid amidases of the three microorganisms and the *R*-stereoselective amino acid amidase from *Arthrobacter* sp. NJ-26. While these amidases show a wide

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Abbreviations: LaaA, L-amino acid amidase; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole.

Enzymes: acylamide amidohydrolases (EC 3.5.1.4); proline iminopeptidases (PIP, EC 3.4.11.5).

(Received 9 January 2004, revised 16 February 2004, accepted 23 February 2004)

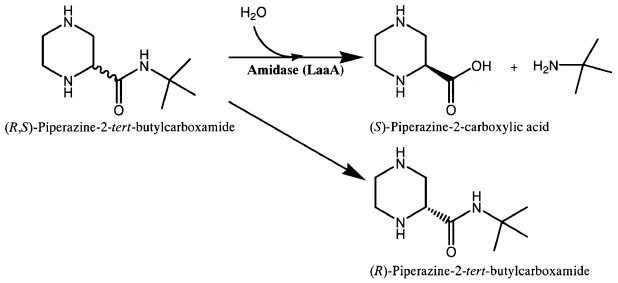

Fig. 1. Stereoselective hydrolysis of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide by the amidase (LaaA) from *P. azotoformans* IAM 1603.

variety of substrate specificities, there is no report on the hydrolysis of amides containing a bulky substituent at the leaving group, such as *tert*-butylcarboxamide. This inability to hydrolyze the bulky amides hindered the wide use of amidases for the production of complex compounds.

Enantiomerically pure piperazine-2-carboxylic acid and its *tert*-butylcarboxamide derivative are important chiral building blocks for some pharmacologically active compounds such as *N*-methyl-D-aspartate antagonist for glutamate receptor [17], cardioprotective nucleoside transport blocker [18] and HIV protease inhibitor [19]. (*S*)-Piperazine-2-carboxylic acid has been prepared by kinetic resolution of racemic 4-(*tert*-butoxycarbonyl)piperazine-2-carboxamide with leucine aminopeptidase [18] or racemic piperazine-2-carboxamide with *Klebsiella terrigena* DSM9174 cells [20]. There is no report on the kinetic resolution of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide.

In this study, we screened for microorganisms that can hydrolyze (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide and found the hydrolytic (amidase) activity in *Pseudomonas azotoformans* IAM 1603. The amidase purified from cells of the strain hydrolyzed *S*-stereoselectively (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide to form (*S*)-piperazine-2-carboxylic acid (Fig. 1). The gene coding for the enzyme was isolated and expressed in *Escherichia coli* host. The recombinant protein was purified and characterized, and found to be a novel L-stereoselective amino acid amidase, LaaA. This is the first report revealing the primary structure of L-amino acid amidase.

Materials and methods

Bacterial strains, plasmids and culture conditions

P. azotoformans IAM (Culture collection of the Institute of Applied Microbiology) 1603 was used as the source of enzyme and chromosomal DNA. *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*Δ M15]) was used as a host for the recombinant plasmids. Plasmids pBluescriptII SK(–) (Toyobo, Osaka, Japan), pUC19 (Takara Shuzo, Kyoto, Japan) and pT7-Blue (Takara Shuzo) were used as cloning vectors. *P. azotoformans* IAM 1603 was cultivated at 30 °C on BM medium containing 10 g Bacto nutrient broth (Difco), 10 g disodium DL-malate *n*-hydrate, 3 g K_2HPO_4, 1 g KH_2PO_4, 0.05 g $MgSO_4 \bullet 7H_2O$, 0.01 g $FeSO_4 \bullet 7H_2O$, 0.01 g $MnCl_2 \bullet 4H_2O$, 0.01 g $CoCl_2 \bullet 6H_2O$, $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ in 1 litre distilled water, pH 7.0. Recombinant *E. coli* JM109 was cultured at 37 °C on Luria–Bertani medium [21] containing 80 μg·ml^{-1} of ampicillin. To induce the gene under the control of the *lac* promoter, isopropyl-thio-β-D-galactoside was added to a final concentration of 0.5 mM.

Purification of the amidase from *P. azotoformans* IAM 1603

P. azotoformans IAM 1603 was subcultured at 30 °C for 16 h in a test tube containing 5 mL BM medium. The subculture (5 mL) was then inoculated into a 2 L Sakaguchi flask containing 500 mL BM medium. The cultivation was carried out at 30 °C for 8 h with reciprocal shaking. All purification steps were performed at a temperature lower than 5 °C. The buffer used was potassium phosphate (pH 7.0) containing 0.1 mM dithiothreitol and 5 mM 2-mercaptoethanol. The protein content of the eluates from column chromatography was monitored by absorbance at 280 nm. Cells (125 g, wet weight) from 25 L of BM medium were harvested by centrifugation (10 000 ***g*** at 4 °C) and suspended in 0.1 M buffer. The cell suspension was disrupted with an ultrasonic oscillator (19 kHz insonator model 201M: Kubota, Tokyo, Japan). The sonicate was centrifuged at 15 000 ***g*** for 20 min at 4 °C, and the resulting supernatant was used as the cell-free extract. The cell-free extract was dialyzed for 12 h against three changes of 10 mM buffer. The dialyzed enzyme solution was then applied to a column (5 × 20 cm) of DEAE-Toyopearl 650M (Tosoh Corp., Tokyo, Japan) previously equilibrated with 10 mM buffer. After the column had been washed with 2 L of 10 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.5 M, 1.5 L each) in 10 mM buffer. The active fractions were combined and then brought to 30% ammonium sulfate saturation and applied to a column (2.5 × 20 cm) of Butyl-Toyopearl 650M (Tosoh Corp.) previously equilibrated with 10 mM buffer 30% saturated with ammonium sulfate. After the column had been washed with 500 mL of the same buffer, the enzyme was eluted with a linear gradient of ammonium sulfate (30–0% saturation, 500 mL each) in 10 mM buffer. The active fractions were combined and dialyzed against 10 L of 10 mM buffer for 12 h. The dialyzed enzyme was applied to a column (1.5 × 8 cm) of Gigapite (Seikagaku Kogyo, Tokyo, Japan) previously equilibrated with 10 mM buffer. After the column had been washed with 50 mL of 10 mM buffer, the enzyme was eluted with a linear gradient of buffer (0.01–1 M, 50 mL each). The active fractions were combined, concentrated with Centriprep-10 (Millipore Corp., MA, USA) and dialyzed against 10 L of 10 mM buffer for 12 h. The dialyzed enzyme was applied to a Superdex 200 HR 26/60 column (Amersham Biosciences K.K., Tokyo, Japan) previously equilibrated with 10 mM buffer containing 150 mM NaCl and eluted with the same buffer. The active fractions were collected and dialyzed against 10 L of 10 mM buffer for 12 h. The dialyzed enzyme was applied to a MonoQ HR 5/5 column (Amersham Biosciences K.K.) previously equilibrated with 10 mM buffer and then eluted with a linear gradient of NaCl (0–0.2 M) in 10 mM buffer. The active fractions were combined, concentrated with Centricon-10 (Millipore Corp.), and submitted to electrophoresis on a nondenaturating polyacrylamide gel, AE-6000 from Atto (Tokyo, Japan). To locate the enzymatic activity,

the gel was divided into aliquots with 5 mm width and 10 mM buffer was added to each gel slice. The protein band corresponding to the enzymatic activity was used for N-terminus and internal amino acid sequencing. The sequencing was carried out by APRO Science (Tokushima, Japan).

Cloning of the *P. azotoformans* IAM 1603 amidase gene (*laaA*)

For routine work with recombinant DNA, established protocols were used [21]. Restriction endonucleases were purchased from Takara Shuzo and alkaline phosphatase from shrimp was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chromosomal DNA was prepared from *P. azotoformans* IAM 1603 by the method of Misawa *et al*. [22]. Oligonucleotide primers were synthesized on the basis of the amino acid sequences of the N-terminal and internal peptides. The amino acid sequence Met-Glu-Phe-Ile-Glu-Lys-Ile was used to model the oligodeoxynucleotide pool 5′-ATGGAGTTCATCGAGAA GATC-3′ (sense strand), and Ala-Ser-Gly-His-Ala-Val-Ile to model 5′-GATSACSGCGTGSCCSSWSGC-3′ (antisense strand) (S = C or G and W = A or T). PCR amplification was performed with these primers, using Expand™ high fidelity PCR system from Roche Diagnostics GmbH. The reaction mixture for the PCR contained 50 μL Expand HF buffer with 1.5 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, the sense and antisense primers each at 1 μM concentration, 2.5 U Expand HF PCR system enzyme mix and 0.5 μg of chromosomal DNA from *P. azotoformans* IAM 1603 as a template. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (initial cycle 2 min 30 s), an annealing step at 55 °C for 30 s and an elongation step at 72 °C for 2 min. The PCR product (186 bp) was cloned into pT7-Blue vector in *E. coli* and was used as a probe for the amidase-encoding gene, *laaA*, of *P. azotoformans* IAM 1603. Chromosomal DNA of *P. azotoformans* IAM 1603 was completely digested with *Fba*I. Southern hybridization showed an ≈ 2.1 kb band from *Fba*I digestion that hybridized with the probe. DNA fragments of 2.0–2.2 kb size range of *Fba*I digestion were recovered from 0.7% (w/v) agarose gel by use of QIAquick™ gel extraction kit from QIAGEN (Tokyo, Japan) and ligated into *Bam*HI-digested and alkaline phosphatase-treated pBluescript II SK(–) using Ligation Kit version 2 from Takara Shuzo. *E. coli* JM109 was transformed with recombinant plasmid DNA by the method of Inoue *et al*. [23] and screened for the existence of the *laaA* gene by colony hybridization with the probe. A positive *E. coli* transformant carried a plasmid, designated pSTB10.

DNA sequence analysis

An automatic plasmid isolation system PI-100 (Kurabo, Osaka, Japan) was used to prepare the double-stranded DNAs for sequencing. The plasmid pSTB10 was used as a sequencing template. Nested unidirectional deletions were generated with the Kilo-Sequence deletion kit (Takara Shuzo). Nucleotide sequencing was performed using the dideoxynucleotide chain-termination method [24] with M13 forward and reverse oligonucleotides as primers. Sequencing reactions were carried out with a Thermo Sequenase™ cycle sequencing kit and dNTP mixture with 7-deaza-dGTP from Amersham Biosciences K.K., and the reaction mixtures were run on a DNA sequencer 4000 L (Li-cor, Lincoln, NE, USA). Both strands of DNA were sequenced. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB087498. Amino acid sequences were compared with the BLAST program [25].

Expression of the *laaA* gene in *E. coli*

A modified DNA fragment coding for the amidase was obtained by PCR. The reaction mixture for the PCR contained, in 50 μL, 10 mM Tris/HCl, pH 8.85, 25 mM KCl, 2 mM $MgSO_4$, 5 mM $(NH_4)_2SO_4$, each dNTP at a concentration of 0.2 mM, a sense and an antisense primer each at 1 μM concentration, 2.5 U *Pwo* DNA polymerase and 200 ng plasmid pSTB10 as a template. Thirty cycles were performed, each consisting of a denaturing step at 94 °C for 30 s (initial cycle 2 min 30 s), an anealing step at 55 °C for 30 s and an elongation step at 72 °C for 2 min. The sense primer contained a *Hin*dIII recognition site (underlined sequence), a ribosome-binding site (double underlined sequence), a TAG stop codon (lowercase letters) inframe with the *lacZ* gene in pUC19, and spanned positions 676–726 in the sequence of GenBank accession number AB087498. The antisense primer contained an *Xba*I site (underlined sequence) and corresponded to the sequence ranging from 1632 to 1654. The two primers were as follows: sense primer, 5′-CGATCCAAGCTTTAAGGAGG AAtagGAAATGGAATTCATCGAAAAAATCCG-3′ antisense primer, 5′-TGCATCCATCTAGAGCATTCA GC-3′. The amplified PCR product was digested with *Hin*dIII and *Xba*I, separated by agarose gel electrophoresis, and then purified with QIAquick™ gel extraction kit. The amplified DNA was inserted downstream of the *lac* promoter in pUC19, yielding pSTB20, and then used to transform *E. coli* JM109 cells.

Purification of the amidase from *E. coli* transformant

E. coli JM109 harboring pSTB20 was subcultured at 37 °C for 12 h in a test tube containing 5 mL Luria–Bertani medium supplemented with ampicillin. The subculture (5 mL) was then inoculated into a 2 L Erlenmeyer flask containing 500 mL Luria–Bertani medium supplemented with ampicillin and isopropyl thio-β-D-galactoside. After a 12 h incubation at 37 °C with rotary shaking, the cells were harvested by centrifugation at 8000 ***g*** for 10 min at 4 °C and washed with 0.9% (w/v) NaCl. All the purification procedures were performed at a temperature lower than 5 °C. The buffer used throughout this purification was Tris/HCl buffer, pH 8.0. Washed cells from 2.5 L culture were suspended in 100 mM buffer and disrupted by sonication for 10 min. For the removal of intact cells and cell debris, the sonicate was centrifuged at 15 000 ***g*** for 20 min at 4 °C. After centrifugation, the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 50–70% saturation was collected by centrifugation and dissolved in 10 mM buffer. The resulting enzyme

solution was dialyzed against 10 L of the same buffer for 24 h. The dialyzed solution was applied to a column (1.5 × 13 cm) of DEAE-Toyopearl 650M previously equilibrated with 10 mM buffer. After the column had been washed thoroughly with 10 mM buffer, the enzyme was eluted with 100 mL 10 mM buffer containing 50 mM NaCl. The active fractions were then brought to 30% ammonium sulfate saturation and added to a column (1.5 × 3 cm) of Butyl-Toyopearl 650M equilibrated with 10 mM buffer 30% saturated with ammonium sulfate. After the column had been washed with the same buffer, followed by 10 mM buffer 15% saturated with ammonium sulfate, the active fractions were eluted with 10 mM buffer 10% saturated with ammonium sulfate. The active fractions were combined and used for characterization.

Enzyme assay

During the purification of the amidase from *P. azotoformans* IAM 1603, the enzyme assay was carried out with (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide as a substrate. The reaction mixture (0.1 mL) contained 10 μmol potassium phosphate buffer (pH 7.0), 5.4 μmol (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide and an appropriate amount of the enzyme. The reaction was performed at 30 °C for 5 h and piperazine-2-carboxylic acid formed was derivatized with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) by the addition of 100 μL 0.1% NBD-Cl in methanol, 100 μL 0.1 M $NaHCO_3$ and 500 μL H_2O to the reaction mixture. After incubation at 55 °C for 1 h, the amount of derivatized piperazine-2-carboxylic acid was determined with a Waters 600E HPLC apparatus equipped with an ODS-80Ts column (4.6 × 150 mm) (Tosoh Corp.) at a flow rate of 0.6 $mL \cdot min^{-1}$, using the solvent system methanol/5 mM H_3PO_4 (2 : 3, v/v). The eluate was detected spectrofluorometrically with an excitation wavelength of 503 nm and an emission wavelength of 541 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol piperazine-2-carboxylic acid per min from (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide under the above conditions. On the other hand, L-prolinamide was used as a substrate during the purification and characterization of recombinant amidase from *E. coli* transformant. The standard reaction mixture (1 mL) contained 100 μmol Tris/HCl buffer (pH 8.0), 20 μmol L-prolinamide hydrochloride and an appropriate amount of the enzyme. The reaction was performed at 30 °C for 5 min and stopped by the addition of 1 mL ethanol. The amount of L-proline formed in the reaction mixture was determined with the HPLC apparatus equipped with Sumichiral OA-5000 column (4.6 × 150 mm) from Sumika Chemical Analysis Service (Osaka, Japan) at a flow rate of 1.0 $mL \cdot min^{-1}$, using the solvent system of 2 mM $CuSO_4$. Absorbance of the eluate was monitored at 254 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol L-proline per min from L-prolinamide under the above conditions. Protein was determined by the method of Bradford [26] using BSA as standard. Enzyme activity toward other amino acid amides and dipeptides was determined by measuring the production of amino acids. Amino acid amides and peptides were purchased from Bachem (Bubendorf, Switzerland), Sigma (Tokyo, Japan) and Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). The amounts of (*R*,*S*)-piperidine-2-carboxylic acid (D,L-pipecolic acid), L-alanine, (*R*,*S*)-piperazine-2-carboxylic acid, L-serine, L-arginine, glycine and L-lysine were quantitatively assayed by HPLC as described for the L-proline. The amounts of L-threonine, L-asparagine, L-glutamine, L-valine and D-proline were assayed by HPLC using the solvent system 2 mM $CuSO_4$/methanol (17 : 3, v/v). The amounts of L-methionine, L-leucine, L-isoleucine and L-aspartic acid were assayed by HPLC using the solvent system 2 mM $CuSO_4$/methanol (7 : 3, v/v). The amounts of L-histidine and L-glutamic acid were assayed by HPLC using the solvent systems 2 mM $CuSO_4$/isopropanol 19 : 1 (v/v) and 17 : 3 (v/v), respectively. The amounts of L-phenylalanine, L-tryptophan and L-tyrosine were assayed by HPLC on an ODS-80Ts column (4.6 × 150 mm) at a flow rate of 0.7 $mL \cdot min^{-1}$ using the solvent system methanol/5 mM H_3PO_4 (1 : 4, v/v). Absorbance of the eluate was monitored at 254 nm. The enzyme activity toward L-proline-*p*-nitroanilide was assayed by the formation of *p*-nitroaniline. A reaction mixture (1.0 mL) containing 5 μmol L-proline-*p*-nitroanilide, 100 μmol Tris/HCl buffer (pH 8.0) and the enzyme, was monitored by the change in absorbance at 405 nm with a Hitachi U-3210 spectrophotometer.

Analytical measurements

To estimate the molecular mass of the enzyme, the sample (10 μg) was subjected to a TSK G-3000 SW column (0.75 × 60 cm; Tosoh Corp.) on an HPLC system at a flow rate of 0.6 $mL \cdot min^{-1}$ with 0.1 M sodium phosphate (pH 7.0) containing 0.1 M Na_2SO_4 at room temperature. Absorbance of the eluate was monitored at 280 nm. The molecular mass of the enzyme was then calculated from the relative mobility compared with those of the standard proteins glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa) (products of Oriental Yeast Co., Tokyo, Japan). SDS/PAGE analysis was performed by the method of Laemmli [27]. Proteins were stained with Brilliant blue G and destained in ethanol/acetic acid/water (3 : 1 : 6, v/v/v).

Results

Purification of the amidase from *P. azotoformans* IAM 1603

An amidase activity versus (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide was detected in *P. azotoformans* IAM 1603. Various nitrogen and carbon sources in the culture media were tested, and the highest activity was obtained after culture in an optimized medium (BM medium) containing Bacto nutrient broth and DL-malate. HPLC analysis with Sumichiral OA-5000 column showed that the *P. azotoformans* IAM 1603 cells acted on (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide to produce (*S*)- and (*R*)-piperazine-2-carboxylic acid, with rather preferred (*S*)-form (Fig. 2A). To investigate the stereoselectivity of the hydrolytic activity toward the substrate, the amidase was purified from the cell free extract of *P. azotoformans* IAM 1603 as described in Materials and methods. From the DEAE-Toyopearl

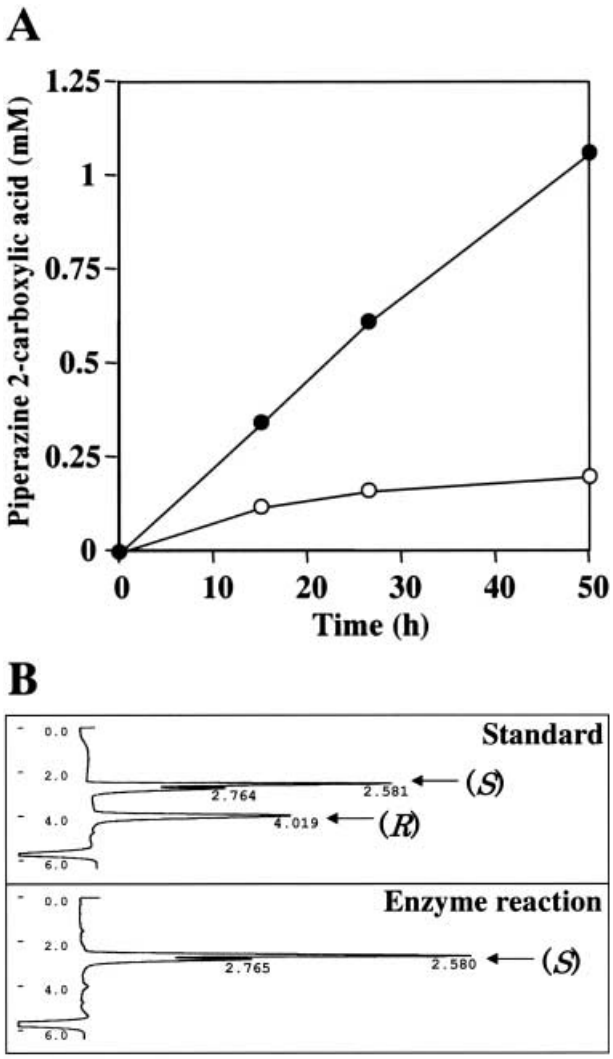

Fig. 2. Hydrolysis of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide by cells of *P. azotoformans* IAM 1603 and stereochemical analysis of piperazine-2-carboxylic acid produced by the purified amidase. (A) *P. azotoformans* IAM 1603 was cultivated in 200 mL of BM medium for 12 h at 30 °C. The cells were then harvested, washed with 0.9% NaCl and suspended in 3 mL of 0.1 M of potassium phosphate (pH 7.0). The reaction mixture contained 10 mM of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide, 150 μL of the cell suspension and 0.1 M of potassium phosphate (pH 7.0) in a total volume of 200 μL, and was incubated at 30 °C. The reaction was stopped at the specific time and the concentration of each enantiomer of piperazine-2-carboxylic acid formed was determined using HPLC with a Sumichiral OA-5000 column as described in Materials and methods. Symbols: ●, (*S*)-piperazine-2-carboxylic acid; ○, (*R*)-piperazine-2-carboxylic acid. (B) The reaction mixture contained 10 mM of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide, 10 μg of the purified amidase and 0.1 M of potassium phosphate (pH 7.0) in a total volume of 200 μL, and was incubated at 30 °C for 10 h. The stereochemistry of the piperazine-2-carboxylic acid formed was determined using HPLC with a Sumichiral OA-5000 column as described in Materials and methods.

column chromatography, two amidase fractions active on (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide were obtained (data not shown). One of the fractions hydrolyzed the substrate *S*-stereoselectively to produce (*S*)-piperazine-2-carboxylic acid, and the other hydrolyzed it nonselectively to produce (*R,S*)-piperazine-2-carboxylic acid. The (*S*)-selective fraction was further purified with a recovery of 0.19% (Table 1). Although the final preparation from the MonoQ column chromatography appeared to be a single band on SDS/PAGE with a molecular mass of ≈ 34 kDa, native polyacrylamide gel electrophoresis showed that the sample still contained some contaminated proteins. After the native polyacrylamide gel electrophoresis, enzymatic activity was located by dividing the gel to assay the activity. The corresponding protein was submitted to N-terminal and internal amino acid sequencing, yielding the following result: MEFIEKIREG for N-terminal and DVAASGH AVI for internal sequences.

Table 1. Purification of the *S*-stereoselective amidase from *P. azotoformans* IAM 1603. (*R*,*S*)-Piperazine-2-*tert*-butylcarboxamide was used as a substrate for total activity and specific activity.

Step	Total protein (mg)	Total activity (mU)	Specific activity ($mU \cdot mg^{-1}$)	Yield (%)
Cell free extract	11200	59.2	5.27×10^{-3}	100
DEAE-Toyopearl	420	15.1	3.57×10^{-2}	25.4
Butyl-Toyopearl	56.2	7.24	0.128	12.2
Gigapite	7.02	1.21	0.171	2.03
Superdex HR26/60	2.10	0.85	0.405	0.14
MonoQ HR5/5	0.123	0.11	0.894	0.19

Cloning of the amidase gene

The oligonucleotide primers used for cloning of the amidase gene by PCR were based on the N-terminal and internal amino acid sequences of the purified amidase from *P. azotoformans* IAM 1603. PCR with the primers and the chromosomal DNA prepared from the strain yielded an amplified 186 bp DNA. Nucleotide sequencing of the DNA fragment revealed that the fragment contained the two amino acid sequences derived from the fragments of purified amidase. Using Southern hybridization with the 186 bp probe, a 2.1 kb *Fba*I signal was obtained. From a genomic *Fba*I DNA library in *E. coli* JM109, a clone containing a plasmid that carried a 2.1 kb insert could be isolated. The plasmid named pSTB10 was used to generate nested deletion plasmids for the determination of the nucleotide sequence. The nucleotide sequence determined was found to be 2104 bp long and two ORFs, ORF1 and ORF2, were present in this region. An amino acid sequence deduced from the ORF2 contained the sequences determined by peptide sequencing, indicating that the ORF2 codes for the amidase. ORF2 was designated *laaA*. The structural gene consists of 930 bp and codes for a protein of 310 amino acids (molecular mass 34 514 Da). A potential ribosome-binding site (AGGG) was located just 7 nucleotides upstream from the start codon ATG, and there was a palindromic sequence suggesting a termination structure downstream from the TGA stop codon of the gene. In the region of DNA upstream of the *laaA* translational start codon, GTTACT and TATCGT sequences relating to the

−35 and −10 consensus promoter regions, respectively, were identified. Alignment by the protein databases using the BLAST program showed that the deduced primary structure of amidase is similar to those of putative proline iminopeptidases from *Pseudomonas syringae* (71.3% identical over 293 amino acids, TrEMBL accession number Q87WK6), *Sinorhizobium meliloti* (66.2% identical over 290 amino acids [28], TrEMBL accession number Q92M42), *Xanthomonas axonopodis* (63.8% identical over 290 amino acids [29], TrEMBL accession number Q8PIB1), *Xanthomonas campestris* (63.4% identical over 290 amino acids [29], TrEMBL accession number Q8P6Z8), *Mesorhizobium loti* (58.1% identical over 291 amino acids [30], PRF accession number 2705259DR), *Salmonella typhimurium* (42.6% identical over 282 amino acids [31], TrEMBL accession number Q8ZPP7) and *Lactobacillus plantarum* (35.6% identical over 292 amino acids [32], TrEMBL accession number Q890D8) and functionally characterized proline iminopeptidases from *Lactobacillus delbrueckii* ssp. *lactis* (37.1% identical over 294 amino acids [33], Swiss Prot accession number PIP_LACDL), *Lactobacillus helveticus* (35.9% identical over 295 amino acids [34], Swiss Prot accession number PIP_LACHE) and *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 397 (35.7% identical over 297 amino acids [35], PRF accession number 2105330A). Figure 3 shows the alignment of the primary structures of the amidase, LaaA, from *P. azotoformans* IAM1603, putative proline iminopeptidase from *P. syringae* and functionally characterized proline iminopeptidase from *L. delbrueckii* ssp. *lactis*. The consensus motif (Gly-X-Ser111-X-Gly-Gly) surrounding the catalytic serine of the proline iminopeptidases family was conserved in LaaA sequence. Asp251 and His278 constituting the probable catalytic triad [36–38] with the Ser111 were also present in the sequence. When the other ORF, ORF1, contained in plasmid pSTB10 locating upstream of the *laaA* ORF, was compared with other sequences in the databases, it was observed that its deduced amino acid sequence showed similarity to those of the following transcriptional regulator proteins: hypothetical LuxR family protein from *P. syringae* (65.8% identical over 202 amino acids, TrEMBL accession number Q87WK7), hypothetical protein SMc04032 from *S. meliloti* (46.0% identical over 202 amino acids [28], TrEMBL accession number Q92M41), hypothetical AhyR/AsaR family protein from *X. axonopodis* (46.8% identical over 201 amino acids [29], TrEMBL accession number Q8PIB0), hypothetical AhyR/AsaR family protein from *X. campestris* (45.9% identical over 205 amino acids [29], TrEMBL accession number Q8P6Z7), hypothetical LuxR family protein from *Rhodopseudomonas palustris* (31.2% identical over 189 amino acids, GenBank accession number BX572594), VanR from *Vibrio anguillarum* (30.1% identical over 193 amino acids [39], Swiss Prot accession number VANR_VIBAN), BafR from *Burkholderia ambifaria* (29.6% identical over 199 amino acids, TrEMBL accession number Q9AER1), hypothetical protein from *Bradyrhizobium japonicum* (29.5% identical over 190 amino acids [40], TrEMBL accession number Q89VI3), MupR from *Pseudomonas fluorescens* (26.8% identical over 194 amino acids [41], PRF accession number 2801295B) and BviR from *Burkholderia cepacia* (27.8% identical over 198 amino acids [42], TrEMBL accession number Q9AHP7). ORF1 was designated *laaR*. Comparison of the deduced amino acid sequences of the *P. azotoformans laaR* and its homologous genes indicated that the ORF1 lacks its 5′ terminus part, probably coding for about 50 amino acid residues.

Production of the LaaA in *E. coli*

The direction of the *laaA* gene was same as that of the *lac* promoter in the plasmid, pSTB10. However, the *E. coli* transformant harboring pSTB10 showed no activity towards the substrates such as (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide, L-prolinamide and L-proline-*p*-nitroanilide, irrespective of the addition of isopropyl thio-β-D-galactoside to the culture medium. To express the *laaA* gene in *E. coli*, we improved the sequence upstream

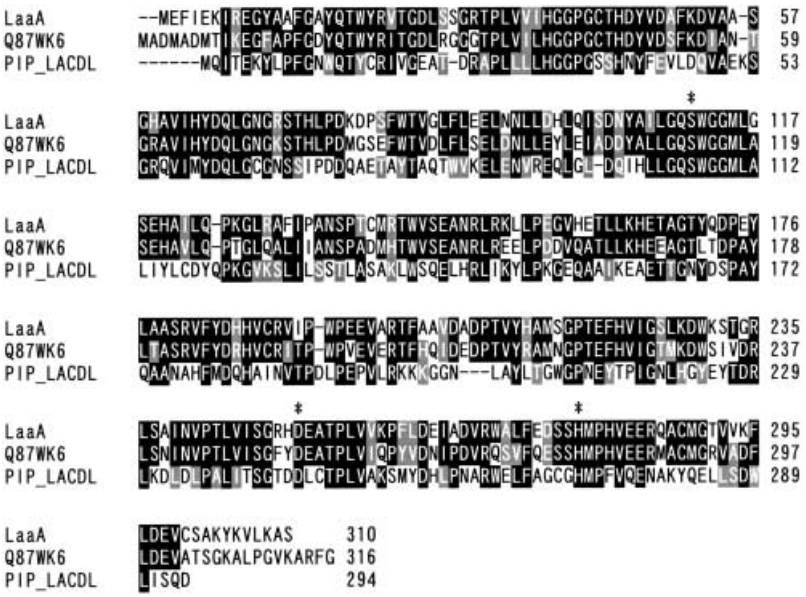

Fig. 3. Comparison of the amino acid sequences of the amidase (LaaA) from *P. azotoformans* IAM 1603 and other homologous proteins. Identical and conserved amino acids among the sequences are marked in black and in gray, respectively. Dashes indicate the gaps introduced for better alignment. LaaA, amidase from *P. azotoformans* IAM 1603; Q87WK6, putative proline iminopeptidase from *Pseudomonas syringae*; PIP_LACDL, proline iminopeptidase from *Lactobacillus delbrueckii* ssp. *lactis*. Three residues, serine, aspartic acid and histidine that constitute the putative catalytic triad are marked by asterisks.

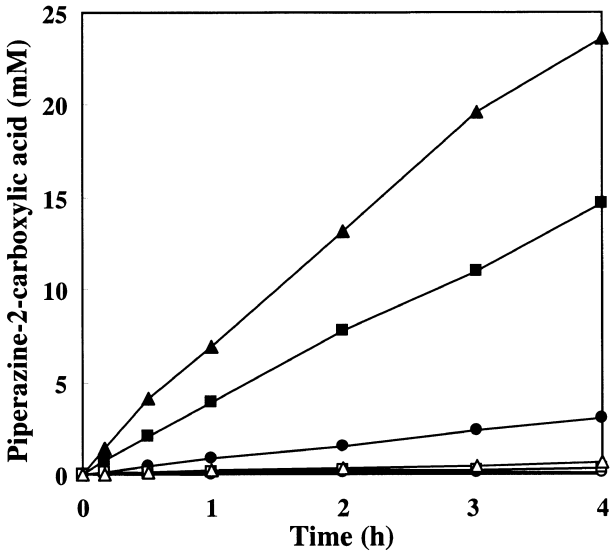

Fig. 4. Stereoselective hydrolysis of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide by cells of *E. coli* JM109/pSTB20. The reaction mixture contained 0.2 M of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide, washed *E. coli* cells prepared from the culture broth after a 12 h cultivation and 0.1 M of Tris/HCl (pH 8.0) in a total volume of 100 μL, and was incubated at 30 °C. The reaction was stopped at the specific time and the concentration of piperazine-2-carboxylic acid formed was determined as described in Materials and methods. Symbols: ●, (*S*)-acid formed with cells (0.28%,w/w); ■, (*S*)-acid formed with cells (1.41%,w/w); ▲, (*S*)-acid formed with cells (2.83%,w/w); ○, (*R*)-acid formed with cells (0.28%,w/w); □, (*R*)-acid formed with cells (1.41%,w/w); △, (*R*)-acid formed with cells (2.83%,w/w).

from the ATG start codon by PCR, with plasmid pSTB10 as a template as described in Materials and methods. The resultant plasmid, pSTB20, in which the *laaA* gene was under the control of the *lac* promoter of pUC19 vector, was introduced into *E. coli* JM109 cells. A protein corresponding to the predicted molecular mass of 34 kDa was produced when the *lac* promoter was induced by isopropyl thio-β-D-galactoside (data not shown). When *E. coli* JM109 harbouring pSTB20 was cultivated in Luria–Bertani medium supplemented with ampicillin and isopropyl thio-β-D-galactoside for 12 h at 37 °C, the level of LaaA activity in the supernatant of the sonicated cell-free extracts of the transformants was 0.026 and 13.2 units·mg^{-1} with (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide and L-prolinamide as substrates, respectively. The cell reaction with 0.2 M of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide was carried out by using the various concentrations of *E. coli* cells (0.28%, 1.41% and 2.83%, w/w) prepared from the 12 h culture (Fig. 4). The *E. coli* cells produced (*S*)-piperazine-2-carboxylic acid with high optical purity (> 95% enantiomeric excess) at all of the reaction times tested.

Purification of the LaaA from *E. coli* transformant

Recombinant LaaA was purified from the *E. coli* JM109 harboring pSTB20 with a recovery of 11.8% by ammonium sulfate fractionation and DEAE-Toyopearl and Butyl-Toyopearl column chromatographies (Table 2). The final

Table 2. Purification of LaaA from *E. coli* JM109 harboring pSTB20. L-Prolinamide was used as a substrate for total activity and specific activity.

Step	Total protein (mg)	Total activity (U)	Specific activity (U·mg^{-1})	Yield (%)
Cell free extract	1020	13400	13.1	100
Ammonium sulfate	354	8720	24.6	65.1
DEAE-Toyopearl	25.5	3900	153	29.1
Butyl-Toyopearl	8.24	1580	192	11.8

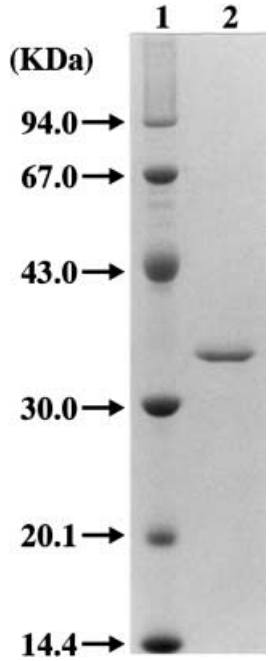

Fig. 5. SDS/PAGE of LaaA. Lane 1, molecular mass standards [phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14,4 kDa)]; lane 2, purified LaaA (5 μg).

preparation gave a single band on SDS/PAGE with a molecular mass of ≈ 34 kDa (Fig. 5). This value is in good agreement with that estimated from the deduced amino acid sequence of the LaaA. The molecular mass of the native enzyme was about 32 kDa according to gel filtration chromatography, indicating that the native enzyme was a monomer. The purified enzyme catalyzed the hydrolysis of L-prolinamide to L-proline at 192 U·mg^{-1} under the standard conditions.

Stability

The purified enzyme could be stored without loss of activity for more than six months at −20 °C in the buffer containing 50% glycerol. The stability of the enzyme was examined at various temperatures. After the enzyme had been preincubated for 5 min in 100 mM Tris/HCl (pH 8.0), a sample of the enzyme solution was taken and the activity was assayed

with L-prolinamide as a substrate under the standard conditions. It exhibited the following activity: 55 °C, 0%; 50 °C, 25%; 45 °C, 81%; 40 °C, 100%; 35 °C, 100%. The stability of the enzyme was also examined at various pH values. The enzyme was incubated at 30 °C for 5 min in the following buffers (final concentration 100 mM): acetic acid/sodium acetate (pH 4.0–6.0), Mes/NaOH (pH 5.5–6.5), potassium phosphate (pH 6.5–8.5), Tris/HCl (pH 7.5–9.0), ethanolamine/HCl (pH 9.0–11.0), glycine/NaCl/NaOH (pH 10.0–13.0). Then a sample of the enzyme solution was taken, and the LaaA activity was assayed with L-prolinamide as a substrate under the standard conditions. The enzyme was most stable in the pH range 6.0–9.5.

Effects of pH and temperature

The optimal pH for the activity of the enzyme was measured in the buffers described above. The enzyme showed maximum activity at pH 9.0. The enzyme reaction was carried out at various temperatures for 5 min in 0.1 M Tris/HCl (pH 8.0), and enzyme activity was found to be maximal at 45 °C. Above 45 °C, it decreased rapidly, possibly because of instability of the enzyme at the higher temperatures.

Effects of inhibitors and metal ions

Various compounds were investigated for their effects on enzyme activity. We measured the enzyme activity under standard conditions after incubation at 30 °C for 5 min with various compounds at 1 mM. The enzyme was completely inhibited by $ZnSO_4$, $ZnCl_2$, $CdCl_2$, $AgNO_3$ and $HgCl_2$ and inhibited 73% by $PbCl_2$, 70% by $NiCl_2$ and 52% by $CoCl_2$. Other inorganic compounds such as LiBr, H_2BO_3, NaCl, $MgSO_4$, $AlCl_3$, KCl, $CaCl_2$, $CrCl_3$, $MnCl_2$, $FeSO_4$, $Fe(NH_4)_2(SO_4)_2$, $CuSO_4$, RbCl, Na_2MoO_4 $(NH_4)_6Mo_7O_{24}$, $SnCl_2$, CsCl and $BaCl_2$ did not influence the activity. The enzyme was completely inhibited by phenylhydrazine, however, other carbonyl reagents such as hydroxylamine, hydrazine, D,L-penicillamine and D-cycloserine were not inhibitory toward the enzyme. Chelating reagents, e.g. *o*-phenanthroline, 8-hydroxyquinoline, ethylenediaminetetraacetic acid and α,α′-dipyridyl had no significant effect on the enzyme. The enzyme was inhibited by thiol reagents such as *p*-chloromercuribenzoate (67% inhibition), iodoacetate (40% inhibition) and *N*-ethylmaleimide (24% inhibition). A serine protease inhibitor, phenylmethanesulfonyl fluoride, a serine/cysteine protease inhibitor, leupeptine and an aspartic protease inhibitor, pepstatin, did not influence the activity.

Substrate specificity

To study the substrate specificity, the LaaA was used to hydrolyze various amino acid amides and dipeptides and the activity was assayed (Table 3). Besides L-prolinamide, the enzyme was active towards L-proline-*p*-nitroanilide (*R*,*S*)-piperidine-2-carboxamide, L-alaninamide and L-methioninamide (*R*,*S*)-piperazine-2-carboxamide. (*R*,*S*)-Piperazine-2-*tert*-butylcarboxamide was, however, hydrolyzed at much lower rates than the above L-amino acid amides. Dipeptides and D-prolinamide were not substrates of the enzyme. The apparent K_m value for L-proline-*p*-nitroanilide was 0.58 mM, whereas the V_{max} value for the substrate was 80.9 $U \cdot mg^{-1}$. Incubation of the LaaA with L-prolinamide and glycine did not yield a dipeptide, L-prolylglycine, suggesting no transpeptidase activity of the enzyme.

Table 3. Substrate specificity of purified LaaA. The activity for L-prolinamide, corresponding to 192 $U \cdot mg^{-1}$, was taken as 100%. The following compounds were not substrates for the amidase: L-argininamide, L-asparaginamide, L-isoasparagine, L-glutaminamide, L-isoglutamine, glycinamide, L-histidinamide, L-lysinamide, L-valinamide, D-prolinamide, L-alanyl-L-alanine, L-alanylglycine, glycylglycine, L-prolyl-L-alanine and L-prolylglycine.

Substrate	Relative activity (%)
L-Prolinamide	100
L-Proline-*p*-nitroanilide	40.9
(*R*,*S*)-Piperidine-2-carboxamide	32.0
L-Alaninamide	10.6
L-Methioninamide	4.2
(*R*,*S*)-Piperazine-2-carboxamide	3.7
L-Phenylalaninamide	0.97
L-Leucinamide	0.46
L-Serinamide	0.43
L-Tryptophanamide	0.20
(*R*,*S*)-Piperazine-2-*tert*-butylcarboxamide	0.20
L-Isoleucinamide	0.17
L-Threoninamide	0.12
L-Tyrosinamide	0.086

Discussion

In this study, we purified an *S*-stereoselective amidase acting on (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide from *P. azotoformans* IAM 1603 and cloned the gene, *laaA*, coding for the enzyme. *E. coli* cells overexpressing the *laaA* gene have been demonstrated to be applicable to the *S*-stereoselective hydrolysis of (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide to produce (*S*)-piperazine-2-carboxylic acid with high optical purity. This is the first example that presents the stereoselective amidase useful for the optical resolution of a racemic amide compound containing bulky substituents at the leaving group.

Sequence analysis of the cloned gene, *laaA*, reveals homology to proline iminopeptidases [PIP, EC 3.4.11.5], which catalyze the removal of N-terminal proline from peptides with high specificity, rather than to the other amidases mentioned in the Introduction, suggesting an evolutionary origin for LaaA from the enzymes involved in peptide degradation. Crystal structures of proline iminopetidases from *X. campestris* pv. *citri* [36] and *Serratia marcescens* [38] have been solved. The enzyme consists of two domains and the larger domain shows the general topology of the α/β hydrolase fold. Ser113, Asp268 and His296 residues (numbering of the residues are based on the enzyme from *S. marcescens*) constituting the catalytic triad are located at the interface of the two domains. Perfect conservation of these residues in the LaaA sequence suggests that LaaA could be categorized as a new member of the family of proline iminopeptidases, and that the

Table 4. Comparison of the characteristics of LaaA from *P. azotoformans* IAM 1603 and bacterial L-amino acid amidases. *p*CMB, *p*-chloromercuribenzoate; DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

	LaaA	L-Aminopeptidase	L-Specific amidase	L-Amino amidase
Origin	*Pseudomonas azotoformans* IAM 1603	*Pseudomonas putida* ATCC 12633	*Ochrobactrum anthropi* NCIMB 40321	*Mycobacterium neoaurum* ATCC 25795
Molecular mass of subunit	34 514 Da	53 000 Da	36 000 Da	40 000 Da
Number of subunits	1	8	2	3 or 4
Optimum pH	9.0	9.5	6.0–8.5	8.0–9.5
pH stability	6.0–9.5			
Optimum temperatrure	45 °C	40 °C	70 °C	50 °C
Heat stability	45 °C		60 °C	55 °C
Inhibitor	Phenylhydrazine, pCMB, iodoacetate, *N*-ethylmaleimide, Zn^{2+}, Ag^{+}, Cd^{2+}, Hg^{2+}	pCMB, DFP, EDTA, PMSF, *o*-phenanthroline, Cu^{2+}, Ca^{2+}	EDTA, *o*-phenanthroline,	DTT, *o*-phenanthroline, iodoacetamide
Activator	No	DTT, Mn^{2+}, Mg^{2+}, Co^{2+}	Zn^{2+}, Mn^{2+}, Mg^{2+}	
Substrate specificity	L-Prolinamide L-Proline-*p*-nitroanilide (*S*)-Piperidine-2-carboxamide L-Alaninamide L-Methioninamide	L-Leucinamide L-Phenylglycinamide L-Methioninamide	L-Prolinamide L-Phenylalaninamide L-Methioninamide L-Phenylglycinamide L-Alaninamide	L-Prolinamide L-Valinamide L-α-Methylvalinamide
Peptidase activity	No	Yes: L-Phe-L-Phe, L-Phe-L-Leu	No	No

catalytic mechanism of LaaA could be analogous to those of the other members. However, LaaA could not act on the peptide substrates such as L-prolyl-L-alanine, L-prolylglycine, L-alanyl-L-alanine, L-alanylglycine and glycylglycine (Table 3). Therefore, LaaA may differ from the other members of the family with respect to its substrate recognition. LaaA was sensitive to heavy metal salts and thiol reagents and rather resistant to serine peptidase inhibitors, suggesting the presence of a possible catalytic cysteine residue. However, these features have also been previously observed in proline iminopeptidases whose catalytic serine residue has been identified by site-directed mutagenesis [43] and crystal structure analysis [36,38].

LaaA was found to have hydrolyzing activity toward L-amino acid amides such as L-prolinamide, L-proline-*p*-nitroanilide, L-alaninamide and L-methioninamide. The enzyme also acted *S*-stereoselectively on (*R*,*S*)-piperidine-2-carboxamide (*R*,*S*)-piperazine-2-carboxamide and (*R*,*S*)-piperazine-2-*tert*-butylcarboxamide. Based on its substrate specificity towards L-amino acid amides, LaaA should be called L-amino acid amidase.

L-Amino acid amidases were previously purified from *P. putida* ATCC 12633 [9], *O. anthropi* NCIMB 40321 [10] and *M. neoaurum* ATCC 25795 [11] and characterized. All of the three enzymes seemed to be metalloenzymes because their activities are inhibited by chelating reagents such as ethylenediaminetetraacetic acid and *o*-phenanthroline and/or activated by divalent cations (Table 4). Comparison of the characteristics of LaaA with those of the other L-amino acid amidases suggests that LaaA is unique not only with respect to its physicochemical characteristics but also concerning its substrate specificity. As the primary sequences of the three amidases have never been reported, LaaA from *P. azotoformans* IAM 1603 is the first L-amino acid amidase whose primary sequence is revealed.

Acknowledgements

We are grateful to S. Iwamoto, R. Kasahara and A. Nakayama (Toyama Prefectural University) for their technical assistance. This work was supported by Grants-in-Aid for Scientific Research (13760076 to H. K.) from JSPS (Japan Society for the Promotion of Science).

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